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Circadian circuits in humans: White matter microstructure predicts daytime sleepiness

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Abstract

The suprachiasmatic nucleus of the hypothalamus is the chief circadian pacemaker in the brain, and is entrained to day-night cycles by visual afferents from melanopsin containing retinal ganglion cells via the inferior accessory optic tract. Tracer studies have demonstrated efferents from the suprachiasmatic nucleus projecting to the paraventricular nucleus of the hypothalamus, which in turn project to first-order sympathetic neurons in the intermedio-lateral grey of the spinal chord. Sympathetic projections to the pineal gland trigger the secretion of the sleep inducing hormone melatonin. The current study reports the first demonstration of potential sympathopetal hypothalamic projections involved in circadian regulation in humans with *in vivo* virtual white matter dissections using probabilistic diffusion tensor imaging (DTI) tractography. Additionally, our data shows a correlation between individual differences in white matter microstructure (measured with fractional anisotropy) and increased daytime sleepiness (measured with the Epworth Sleepiness Scale (ESS, Johns, 1991)). Sympathopetal connections with the hypothalamus were virtually dissected using designated masks in the optic chiasm, which served as an anatomical landmark for retinal fibres projecting to the suprachiasmatic nucleus, and a waypoint mask on the lateral medulla, where hypothalamic projections to the sympathetic nervous system

traverse in humans. Sympathopetal projections were demonstrated in each hemisphere in twenty-six subjects. The tract passed through the suprachiasmatic nucleus of the hypothalamus and its trajectory corresponds to the dorsal longitudinal fasciculus traversing the periaqueductal region and the lateral medulla. White matter microstructure (FA) in the left hemisphere correlated with high scores on the ESS, suggesting an association between circadian pathway white matter microstructure, and increased daytime sleepiness and decreased arousal.

Keywords: suprachiasmatic nucleus, diffusion tensor imaging, melatonin, circadian rhythms, sleep-wakefulness.

Circadian rhythms are biochemical, physiological and behavioural rhythms that oscillate with a period close to 24 hours and are generated via an internal pacemaker, the suprachiasmatic nucleus of the hypothalamus, located in the rostroventral hypothalamus above the optic chiasm (Fuller & Fuller, 2002). The suprachiasmatic nucleus is considered to be the chief pacemaker for clock genes in the brain in addition to regulating various endocrine, physiological and behavioural circadian rhythms (Hoffmann & Swaab, 1993). Circadian rhythms have been demonstrated in diurnal cycles of core body temperature, and the release of hormones such as melatonin and cortisol (Hoffstra & de Weerd, 2008).

While this characteristic near 24-hour cycle persists in the absence of environmental time-cues (e.g. light), daylight acts as a *zeitgeber* to ensure that it is entrained to the light-dark solar cycle (Fuller & Fuller, 2002). Light signals transduced by melanopsin containing retinal ganglion cells (Gooley et al., 2001) are transmitted to the suprachiasmatic nucleus via the inferior accessory optic tract (Moore et al., 1966). This circuitry allows entrainment of the suprachiasmatic nucleus, which regulates the sleep-wake cycle through the sleep inducing hormone melatonin, to light-dark cycles.

Light induced inhibition of melatonin secretion is controlled through a multi-synaptic pathway from the suprachiasmatic nucleus to the pineal gland (Moore & Card, 1986; Teclemariam-Mesbah et al., 1999). The suprachiasmatic nucleus projects to the paraventricular nucleus including its autonomic parts such as the dorsal paraventricular nucleus (pe) and the medial parvocellular part (mp). Direct hypothalamic projections from the paraventricular nucleus in turn synapse in the intermediolateral column in the first segment of the thoracic spinal cord, where first order sympathetic neurons further project to the superior cervical ganglion. Second order sympathetic fibres project from the superior cervical ganglia to the pineal gland (Kappers, 1976). Sympathetic fibres reach the pineal gland, entering the apex of the pineal gland from the region of the *tentorium cerebelli* as single or paired *nervi conarii* in humans and rodents. Norepinephrine is released from their terminals forming synapses on the surface of pinealocytes, which thereby convert serotonin into melatonin (Alarma-Estrany & Pintor, 2007). A schematic presentation of this pathway is presented in Figure 1. Our study set out to visualise the descending, sympathopetal component of these hypothalamic projections in human volunteers, and to examine how the microstructure of this pathway may relate to measures of circadian rhythm.

To do so, we used probabilistic DTI tractography to virtually dissect potential descending pathways that may function in regulating diurnal sleep-wake cycles. Our goal was to demonstrate hypothalamic projections to the sympathetic nervous system that receive afferents from the suprachiasmatic nucleus. The chief circadian pacemaker is termed suprachiasmatic, due to its location directly dorsal to the optic chiasm. Since it is small and its margins are not discernable on MRI images, it was not possible to reliably draw a seed mask that was restricted to the suprachiasmatic nucleus. Therefore, to ensure that the connection passed through the suprachiasmatic nucleus, a seed mask was drawn on the optic chiasm (from which the suprachiasmatic nucleus receives visual afferents). A waypoint mask was drawn on the lateral medulla, through which hypothalamic projections to the sympathetics are known to pass (Naidich et al., 2009). Connections between the suprachiasmatic nucleus and the lateral medulla were virtually dissected in two groups of sleep-chronotype individuals; morning and evening types. Individual differences in microstructure (using fractional anisotropy) of the resulting pathways, in addition to physiological measures of circadian rhythms (melatonin, cortisol, heart rate, breathing rate and temperature) were compared between morning and evening sleep types. We successfully demonstrated the posited pathway and provide novel evidence that, although the microstructure of the proposed sympathetic sleep circuit dissected in our sample did not correlate with measures of circadian rhythms, its microstructure (FA) did predict daytime sleepiness.

Our prediction was that measures of circadian rhythm would correlate with microstructure of virtual dissections of the proposed sleep pathway. However, instead, our data shows that daytime sleepiness correlated with microstructure of the sleep pathway. Therefore, we conclude that the strong correlation between microstructure of the sleep pathway and daytime sleepiness offers only evidence for the functional veracity of its role in sleep, but not a putative mechanism by which this is achieved.

Material and Methods

Participants

An initial sample of 27 healthy participants (15 female, age range 21-57) was recruited from the population at Bangor University. The study was advertised through posters, online forums and a participant recruitment website at Bangor University. Potential participants were first required to fill out the Morningness-Eveningness Questionnaire (Horne & Östberg, 1976), Beck's Depression Inventory (Beck, Steer & Brown, 1996), the Epworth Sleepiness Scale (Johns, 1991) and a short questionnaire on lifestyle and sleeping habits that may interfere with circadian rhythms. Questionnaires were sent to participants in the form of online links that were completed online to allow participants to fill out confidential information in private at their own convenience. Each participant was provided with a confidential participant number when filling out online questionnaires to ensure anonymity. Participants were invited to take part in the study if they met the following inclusion criteria: 1) scored either as an 'early bird' (>58) or 'night owl' (<42) on the MEQ, 2) scored below the threshold that would indicate mild to moderate depression on BDI (<9), 3) scored below the threshold that would indicate excessive daytime sleepiness on the ESS (<10) and 4) demonstrated that their sleep type (as indicated by MEQ score) was not due to lifestyle factors such as shift work, heavy alcohol consumption, sleep disorders or use of medication. Participants had no known neurological, psychological, psychiatric, sleep or cognitive impairments. Finally, participants were screened against exclusion criteria related to MR safety. Monetary compensation was provided for participation in MRI scanning. One participant was excluded from the study after failure to adhere to the study instructions. Ethical approval was received from Bangor University's Ethics Review Committee and prior written consent was obtained from all participants. This study conformed to the ethical standards of the Declaration of Helsinki. Participants were informed that their data and personal demographical information would be kept anonymous at all times.

MRI data collection and pre-processing protocol

Magnetic resonance scanner

All imaging data was collected on a Phillips 3 Tesla Achieva magnetic resonance (MR) scanner (Phillips Medical, Best, Netherlands) at the Bangor Imaging Unit at Bangor University.

T1 anatomical scans

High resolution multi-echo T1 weighted images (0.7x0.7x0.7 mm isotropic voxel resolution) were acquired using a 5 echo averaged MP-RAGE sequence (TE = 3.5, 5.1, 6.8, 8.5, 10.2 ms, effective TE = 6.7 ms, TR = 12 ms, TI = 1150 ms, 3D acquisition, FOV = 240 mm X 220 mm X 130 mm, voxel dimensions = 0.7 X 0.7 X 0.7 mm^3).

DTI scans

DWI-EP (diffusion weighted imaging – echo planar) images were collected at 2x2x2mm with the following parameters: b-values = 0 (averaged four volumes) and 2000, b-directions = 61, slices = 76, section thickness = 2mm, TR = 2 s, TE = 35ms. All participants were scanned using a 32-channel head coil.

MRI Data pre-processing

Following data acquisition, the image files for the DTI data and the structural T1 scans were manually converted from DICOM format into NIFTI with dcm2nii (http://www.sph.sc.edu/comd/rorden/mricron/). Subsequent data pre-processing was carried out using the FSL-FDT toolbox (Behrens et al, 2003, 2007; http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/). Diffusion weighted images were corrected for eddy currents and head motion using affine registration to the first b-zero volume. This was carried out to prevent approximate stretches and shears created in the diffusion weighted images that may have been induced by eddy currents in the gradient coils (Behrens et al., 2007). After eddy current correction, diffusion tensor models were fitted at each voxel using the DTI-FIT tool in FSL. Diffusion parameters were calculated using the Markov Chain Monte Carlo

sampling method. The DTI data was then prepared for probabilistic tractography via the BEDPOSTX tool in FSL's FDT toolbox. BEDPOSTX ran with the following parameters: number of fibres modelled per voxel = 3, weight = 1, burning period = 2000). Probabilistic tracking was carried out using the PROBTRACKX tool in FSL's FDT toolbox. PROBTRACKX ran with the following parameters: curvature threshold = .02, number of samples generated per voxel = 5000, number of steps per sample = 2000 and steplength = .05. Prior to running the BEDPOSTX process, the non-diffusion brain image was extracted from the skull (using the FSL brain extraction tool (BET)), and a brain mask was formed. Anatomical T1-weighted scans were brain extracted using the BET-tool, and were registered with the B0 diffusion brain image, using FSL's FLIRT tool. Masks were drawn based on anatomical landmarks in each participant's individual non-diffusion T1-registered brain using FSLView.

Physiological measures of circadian rhythms

Participants additionally provided physiological measures of circadian rhythms including saliva samples to test for melatonin, and measures of heart rate, breathing rate and temperature. In accordance with the Salimetrics ® saliva collection protocol, participants were required to abstain from consuming alcohol, caffeine, nicotine, strenuous exercise, aspirin and ibuprofen during the testing period. Participants were asked to adhere to these restrictions from the evening prior to the testing day until after the final saliva sample was collected. Consumption of food was allowed only up until 30 minutes prior to saliva collection. One participant was excluded from the study after failure to adhere to the study instructions. Heart rate was measured by asking the participant to count the number of pulse beats on their wrist for one minute. Breathing rate was measured as the amount of breaths (one breath counted as one inhale and one exhale) per minute. Disposable oral thermometers were used to measure body temperature. In comparison to circadian hormones (melatonin and cortisol) and temperature, measured via physiologically robust saliva sample assay and oral thermometers, respectively, measures of heart and breathing rate were self counted, but under thorough supervision by the experimenter. Therefore, measures of heart and breathing rate should be considered less rigorous as measures of circadian rhythm.

Procedure

Physiological measures were carried out in the morning (8am) and evening (9pm) on the same day as MRI testing. All MRI data collection was conducted during morning hours (9am-12am). Participants had the option to collect the measures in the privacy of their own home, or to receive assistance from the experimenter at Bangor University. Following the Salimetrics ® saliva collection protocol, ten minutes prior to saliva collection, the participant was required to rinse orally with water only and was asked to sit still in a darkened room with minimal light (<8 Lux). After 10 minutes, participants collected their own saliva sample in a cryovial tube. Tubes were labelled and coded to ensure anonymity, and were immediately stored in a freezer in a Human Tissue Authority licensed lab (at approximate temperature -20 °C) at Bangor University. Following saliva collection, the participant was provided with a disposable thermometer and asked to place it under the tongue for 60 seconds. The participant was then asked to locate their pulse on their wrist and count the number of beats per minute, over one minute. Finally, the number of breaths per minute was recorded. The experimenter recorded the times of saliva, temperature, heart rate and breathing rate collection to ensure that the timings were the same for each participant. Prior to MRI scanning, the participant was provided with a MRI safety-screening questionnaire and interview. Once both the experimenter and the participant were satisfied that it was safe for the participant to be scanned, the scanning session started and lasted approximately 40 minutes.

Virtual dissection of sleep pathways with probabilistic DTI tractography

Probabilistic tractography

The goal of the virtual dissection was to generate connections that traversed the suprachiasmatic nucleus of the hypothalamus through the lateral medulla en route to the sympathetic nervous system in the spinal cord. To demonstrate such hypothalamic projections from the paraventricular nucleus neurons that receive afferents from the suprachiasmatic nucleus, a seed mask was placed in the optic chiasm from which visual afferents project to the suprachiasmatic nucleus via the inferior accessory optic tract. A waypoint mask was placed on the lateral medulla to visualize projections from the paraventricular nucleus of the hypothalamus that synapse on preganglionic first order

sympathetic neurons in the intermediolateral column of the spinal cord.

Connections were generated using FSL's FDT DIFFUSION toolbox in PROBTRACKX (Behrens et al., 2003; Behrens, Johansen-Berg, Jbabdi & Woolrich, 2007) in both hemispheres of each subject's diffusion weighted image using the subject-specific masks manually drawn on: 1) the optic chiasm, 2) the lateral medulla and 3) exclusion regions. Masks were drawn manually (using FSL's FSLVIEW in the FTD DIFFUSION toolbox) on each subject's diffusion space registered T1-weighted anatomical brain image using FSL's FLIRT LINEAR REGISTRATION). Figure 2 shows the optic chiasm and lateral medulla region of interest masks, in addition to an exclusion mask in one representative subject.

The value of each voxel of the resulting connection represented the total number of traces passing through that voxel. We thresholded each voxel so that only voxels that contained at least 10% of the maximum number of traces found in any voxel remained. A study that directly compared diffusion tensor imaging (DTI) tractography with tracers in monkeys has reported that a threshold of 10% is optimal to most reliably reflect the anatomy of tractography compared with tracers (Azadbakht et al. 2015). Connections were generated in both directions (i.e. optic chiasm as seed and lateral medulla as waypoint mask, and vice versa). The two connections generated in each hemisphere were added together (using FSLMATHS), and only voxels that 'overlapped', i.e. were present in both connections, were isolated as a composite connection in each hemisphere of each participant for use as a mask in subsequent analyses of white matter microstructure.

Measurement of FA values and statistical analyses

Voxels in the composite overlap connection in each hemisphere of each participant were used as masks in native diffusion space to calculate, with FSLmaths, the mean fractional anisotropy values of all voxels in each connection for each participant. The composite overlap of the voxels that were present only in connections in both directions were isolated in order to include voxels that would most likely be part of the neural pathway between the suprachiasmatic nucleus and the lateral medulla. We presumed that voxels containing fibres regulating circadian rhythms could also include other fibers projecting from the paraventricular nucleus to the sympathetic nervous system. Since the structural connectivity

of these non-circadian projections were presumed to have no correlation with sleep behaviour, it was important to reduce noise by constraining the size of the connection as much as possible and to exclude, as much as possible, other projections to the sympathetics in the spinal cord, in addition to other adjacent descending tracts in the densely packed brainstem.

For visualization purposes, and for demonstration of anatomical variability across participants, composite, binarized connections for all participants were registered to the same brain space and added together to produce images showing the percent of participants through which the connection passed in each voxel (Figure 3).

Statistical analyses

The first goal of this study was to virtually dissect a connection between the suprachiasmatic nucleus of the hypothalamus and the lateral medulla using diffusion tensor tractography.

The second goal was to test for behavioural and physiological differences between morning and evening sleep type individuals. To simplify this comparison of several sleep and circadian rhythm measures, a single score was computed for each measure (mean concentration of melatonin in pg/ml, and measures of temperature, heart rate and breathing rate) by subtracting the score obtained in the evening testing from that obtained in the morning testing for each participant. Independent samples t-tests were carried out to compare scores of measures of 1) mean concentration of melatonin, 2) temperature, 3) heart rate, 4) breathing rate and 5) white matter microstructure (FA) of the dissected connection between the suprachiasmatic nucleus and the lateral medulla (FA) between morning and evening sleep types. In order to control for multiple comparisons, Bonferroni correction for multiple comparisons was carried out.

The final goal of this study was to attempt to validate that the pathway we dissected with DTI plays a function in regulating sleep. In order to achieve this, mean FA values of the resulting overlap sleep pathways were correlated with measures of sleep behaviour (salivary melatonin and daytime sleepiness) across participants. Firstly, a paired samples t-test was employed to test for differences in FA between dissected pathways in the left and right hemispheres. Pearson correlation was used to test for correlations between microstructure between the suprachiasmatic nucleus and the lateral medulls (FA) and two measures of sleep:

1) melatonin and 2) scores on daytime sleepiness (ESS scores) for each participant in the left and right hemispheres. Bonferonni correction for multiple comparisons was carried out for correlations between pathway FA and 1) melatonin and 2) ESS scores for both the left and right hemispheres.

Results

Virtual dissection between the suprachiasmatic nucleus and the lateral medulla with probabilistic DTI tractography

Virtual dissection of tracts connecting the suprachiasmatic nucleus of the hypothalamus to the lateral medulla were demonstrated bilaterally in 24 participants, and unilaterally in the left hemisphere of one participant and the right hemipshere of another. One participant did not demonstrate the pathway either in the left or the right hemisphere. This connection demonstrates a putative sympathopetal circadian pathway from the hypothalamus that traverses the paraventricular nucleus of the hypothalamus to the intermediolateral column in the spinal cord. Its trajectory connects retino-hypothalamic fibres in the inferior accessory optic tract to the suprachiasmatic nucleus, passes through the paraventricular nucleus of the hypothalamus and thence follows a trajectory corresponding to the dorsal longitudinal fasciculus of Schütz which traverses the dorsal midbrain through the periaqueductal region.

For illustration purposes, Figure 3 presents the common overlap of the composite connection from all participants registered to a common brain space. However, as described below, mean FA was calculated for each individual in each hemisphere in native diffusion space.

Calculation of microstructure with fractional anisotropy

Overlapped tracts for each participant were used as masks on the fractional anisotropy (FA) image for each participant (generated with DTIFIT utility in the FTD toolbox of FSL) to calculate mean FA values for each connection in each individual participant. Paired-samples t-tests demonstrated no significant differences in mean FA between left and right pathways ($t(23) = -.78$, $p = .44$, two-tailed). Table 1 presents FA values of the connection for each participant.

Differences between morning and evening sleep types

An independent samples t-test demonstrated no significant differences in the mean FA averaged across the left and right hemispheres for each participant (a single hemisphere value was used in place of an average for participants in whom the tract was demonstrated in one hemisphere only) between the morning and evening sleep type groups ($t(24) = .55$, $p = .59$). In order to compare physiological results between the groups of morning and evening sleep types, new scores were computed for each measure by subtracting the score that was obtained in the evening testing from the morning testing. Difference scores were computed for the mean concentration of melatonin in pg/ml, and measures of temperature, heart rate and breathing rate. Independent samples t-tests showed no significant differences in melatonin ($t(21) = -1.68$, $p = .12$), cortisol ($t(22) = 1.72$, $p = .1$), breathing rate ($t(22) = 1.68$, $p = .11$ or heart rate ($t(22) = 1.56$, $p = .13$) between the two sleep groups. A significant difference was obtained for temperature between morning and evening types ($t(23) = 2.58$, $p = .017$) with a larger temperature decrease demonstrated for the evening type group. However, this result did not survive the significance cut off of $p < .002$ after Bonferroni correction for multiple comparisons. Figure 4 presents bar graphs and difference scores between morning and evening type sleep groups.

Microstructure of suprachiasmatic nucleus – lateral medulla tract with measures of sleep behaviour

Microstructure (FA) between the suprachiasmatic nucleus and the lateral medulla were correlated with two measures of sleep; 1) melatonin measures and scores on daytime sleepiness as measured by the ESS. Pearson correlation demonstrated no significant correlation between FA of the suprachiasmatic nucleus – lateral medulla connections and mean melatonin difference scores (i.e the amount of diurnal fluctuation in melatonin levels) in the left hemisphere ($r = .3$, $p = .17$, $N = 22$) or the right hemisphere ($r = -.05$, $p = .83$, $N = 22$). Furthermore, no correlations were observed for total melatonin measured (morning and evening measures added) and pathway microstructure in the left ($r = .23$, $p = .28$) or the right ($r = -.07$, $p = .76$) hemispheres. A significant correlation was observed between FA of connections in the left hemisphere and daytime sleepiness as measured by the ESS ($r = .69$, $p < .001$, $N = 25$) (Figure 5). No significant correlation was observed between daytime

sleepiness and FA in the right hemisphere (r = .18, p = .39, N = 25). The effect of FA in the left hemisphere to predict daytime sleepiness was significant after Bonfferoni correction for multiple comparisons (p < .002). Correlations between microstructure (FA) in the left and right were not significant in any of the other measures of circadian rhythm.

Critical consideration of plausibility of virtually dissected pathway

Considering the challenges inherent to probabilistic tractography, we carried out several quality assurance checks to rule out potential false positives for the virtually dissected sleep tracts. Data quality assessment was carried out to ensure that none of the diffusion images were subjected to distortions in the region of the optic chiasm. Although distortions are likely to exist in all areas throughout the brain, bias field distortions are likely to be more extensive in orbitofrontal regions, and although this region is in close proximity to the optic chiasm, our data suggest that this area was unaffected by bias field distortions (Figure 6.) Furthermore, although the seed mask was placed in the optic chiasm, the tract connects in the direction opposite to the orbitofrontal region, thus making the possibility of obtaining the tract as a result of false positive connections due to bias field distortion unlikely. To ensure that the region of interest masks overlapped with the correct corresponding anatomical landmarks, several quality assessment steps were carried out to ensure that the masks corresponded with the DTI data. The T1 structural image was first registered to the diffusion brain, and the masks were drawn on this registered image. Thus, the masks were drawn in diffusion space with the T1 structural registered to diffusion space as an anatomical guide. Furthermore, the masks were then overlaid on the B0 diffusion image to confirm that the masks matched to local anatomical landmarks in diffusion space. Additionally, visualisation of the tensor model in close up along the tract close to seed regions demonstrated that the direction in each voxel were consistent along the tract (Figure 7). Together, the data quality checks above suggest that the virtually dissected tracts were unlikely to have been demonstrated due to spurious connections influenced by distortion.

Discussion

Summary of main findings

In this study, descending white matter pathways postulated to be involved in the regulation of circadian rhythms were virtually dissected in the human brain with the use of probabilistic diffusion tensor imaging tractography. Seed masks were placed in the optic chiasm to ensure that the resultant connections would demonstrate connections between the suprachiasmatic nucleus of the hypothalamus, the chief internal circadian pacemaker, which is located directly above the optic chiasm and which receives retinal afferents via the inferior accessory optic tract. The resulting connections passed through the region of lateral medulla known to transmit hypothalamic projections, originating in the paraventricual nucleus, to pre-ganglionic sympathetic neurons in the intermediolateral column of the spinal cord. Its topography was visualized as a flat trajectory across the meso-diencephalic junction and then descended along the midline through the periaqueductal region to the lateral medulla. The trajectory demonstrated in these virtual dissections corresponds to the anatomy of the dorsal longitudinal fasciculus of Schütz (1891), which contains sympathopetal fibers projecting from parvocellular neurons in the paraventricular nucleus of the hypothalamus to first order preganglionic neurons in the intermediolateral gray column of the spinal cord (Kiernan, 2005).

Comparison of measures of circadian rhythms between morning compared to evening type groups demonstrated no differences in measures of heart rate, melatonin, cortisol, breathing rate and microstructure (FA) of the connections between the suprachiasmatic nucleus and lateral medulla. However, a trend of greater temperature difference between morning and evening measures were observed within the evening type group compared to the morning type group. White matter microstructure of the circadian tract in the left hemisphere predicted daytime sleepiness as measured by the Epworth Sleepiness Scale (Johns, 1991). However no correlations between pathway microstructure and diurnal fluctuations of salivary melatonin was observed. Findings from this study indicated that the microstructure of the virtually dissected pathway connecting the suprachiasmatic nucleus and lateral medulla in the left hemisphere plays a role in modulating sleep-wakefulness.

Anatomical connectivity

Tracer studies in several species have shown that there are direct, sympathopetal projections from the paraventricular nucleus of the hypothalamus that synapse on pre-ganglionic, first order sympathetic neurons in the intermediolateral column of the spinal cord (Cechetto & Saper, 1988, Larsen et al., 1991; Hosoya et al.,1985). There is evidence that these sympathopetal fibers descend through two distinct pathways (Larsen et al, 1998): one coursing along the periaqueductal gray and another through the central pons (Swanson, 1977, Figure 5, p. 351). In humans, the pathway passing through the periaqueductal region is a component of the dorsal longitudinal fasciculus (Kiernan, 2005).
It has not been previously established which of these descending hypothalamo-sympathetic pathways contain fibers from the hypothalamus that transmit signals from the suprachiasmatic nucleus to regulate circadian cycles through sympathetic efferents to the pineal gland. The 'classical model' of neural control of the pineal (Larsen et al., 1998, Figure 1, p. 129) depicts these axons as traversing the centre of the pons.

However, Larsen et al. (1998) specifically examined hypothalamic connections to sympathetic neurons in the intermediolateral column of the spinal cord that were involved in control of pineal gland secretion. They injected a neurotropic alpha herpesvirus (pseudorabies virus) into the pineal gland of rats, and localized viral antigens in infected neurones at various postinoculation intervals. Infected neurons were observed in the superior cervical ganglion, intermediolateral columnn of the thoracic spinal cord, periaqueductal gray, and the paraventricular and suprachiasmatic nuclei of the hypothalamus.

In the current study, we attempted to identify the descending sympathopetal projections from the hypothalamus that transmitted signals from the central circadian pacemaker, the suprachiasmatic nucleus of the hypothalamus. The human pathway demonstrated in the current study traversed the periaqueductal region as a component of the sympathopetal fibers in the dorsal longitudinal fasiculus. This topography is consistent with the trajectory described by Larsen et al. (1998) in rats. Circadian circuitry mediates a primitive function, so it might be expected to be highly conserved during evolution.

Tractography did not demonstrate hypothalamic connections to the sympathetic nervous system that project in the more ventral pathway through the central pontine tegmentum. Since

the seed mask used was the optic chiasm, it is possible that the connection passed through just the part of the paraventricular nucleus that projects through the dorsal longitudinal fasciculus. Given the low spatial resolution of diffusion imaging, however, it seems unlikely that some of the voxels through which the connection passed did not contain fibres that projected through the more ventral route in the pontine tegmentum. It is more likely that the failure to demonstrate the more ventral pathway reflects a limitation of tractography. Tractography demonstrates the vector of anisotropic water diffusion. It provides no information about the direction of axonal projections, or synaptic connections. A connection is only generated in voxels which predominantly contain axons oriented in the same direction. If fibres cross within a voxel perpendicular to another, mean diffusion in that voxel will not be anisotropic. Unlike the dorso-medial pons in which there are dominantly longitudinally oriented fibres, the pontine tegmentum contains both longitudinal (ascending and descending) fibres, and transversely oriented fibres. This may have precluded demonstration of the ventral pathway through the pontine tegmentum in our virtual dissection.

Microstructure of the suprachiasmatic nucleus-lateral medulla connection in the left hemisphere predicts daytime sleepiness

Our results demonstrated that the white matter microstructure (FA) of the suprachiasmatic nucleus-lateral medulla pathway predicted daytime sleepiness, however this effect was only demonstrated in the left hemisphere. Thus, it is important to consider two potential interpretations of this asymmetry.

Firstly, the possibility that hemispheric asymmetry exists for pathways that regulate sleep cannot be ruled out since hemispheric asymmetry is often reported for other functions such as right hemispheric dominance for attention (Hellman & Van Den Bell, 1980; Shulman et al., 2010) or left hemispheric dominance for language (Knight et al., 2000). However, there is no theoretical background to suggest that circuitry that regulates sleep should be lateralised. Therefore, it cannot be concluded based on our data alone that the role of autonomic projections from the suprachiasmatic nucleus in regulating daytime alertness is exclusive to the left hemisphere.

Secondly, our data begs the question of why microstructure (FA) in the left hemisphere predicts daytime sleepiness. The connection between the suprachiasmatic nucleus and the lateral medulla passed through the region of locus coeruleus, which transmits ascending noradrenergic projections that stimulate wakefulness as part of the reticular activating system (Sapper et al., 2005). However, since microstructure (FA) predicted increased daytime sleepiness rather than arousal, it is unlikely that the dissected pathway formed part of the reticular activating system. This provides supporting evidence that the dissected pathway instead forms part of descending sympathetic projections that trigger sleep inducing melatonin secretion, thereby accounting for daytime sleepiness. However, our data did not support our prediction that microstructure correlates with salivary measures of circadian melatonin fluctuation. Therefore, while our data supports the hypothesis that the dissected connection between the suprachiasmatic nucleus and the lateral medulla plays a role in sleep-wakefulness cycles, it does not confirm the hypothesis that this pathway is involved in regulation of circadian melatonin secretion. Future research may confirm laterality differences in suprachiasmatic nucleus-lateral medulla pathway microstructure (FA) as predictor of daytime sleepiness.

One major advantage of DTI tractography over other techniques is that it is a non-invasive method for investigating white matter pathways in the brain. When using probabilistic DTI, it is possible to make judgements on the reliability of the pathways observed (Parker, 2011), and to compare factors that support the existence of a pathway (e.g. high FA value). Therefore, probabilistic DTI is not only a quality assurance tool, but additionally allows for comparison of white matter structural connectivity between brains from different groups of individuals (Parker, 2011). The main contribution of this study is that the sympathetic circadian pathways reported in humans, compares well with that found in the rat brain, pointing to an anatomical representation of the human sympathetic circadian pathway.

Our data presents the first evidence that hypothalamic projections to the sympathetic nervous system in humans play a role in regulating daytime sleepiness. The finding that daytime sleepiness correlated strongly with microstructure (FA) of the streamline was not predicted by our hypothesis; and certainly we had no basis to hypothesize lateralization of the effect to the left hemisphere. Melatonin per se is not sedating in physiological amounts and its role is to trigger sleep. Sleep is induced by the resulting serotonergic projection from the midline raphe in the brainstem. The streamline virtually dissected in this investigation corresponds to

the dorsal longitudinal fasciculus of Schütz and is not consistent with projections from raphe nuclei of the reticular activating system. Moreover, the microstructure (FA) of the streamline did not correlate with melatonin levels. We are not, therefore, able to proffer a putative mechanism accounting for the correlation between microstructure (FA) of the streamline and daytime sleepiness. Nor can we offer an explanation for its laterality to the left hemisphere. We can only conclude that the strong correlation between microstructure and behaviour offers evidence for the veracity of the streamline as a functional anatomical pathway; and suggest that the dorsal longitudinal fasciculus contains projections, in the left hemisphere, that predict daytime sleepiness. We hope that our empirical findings will stimulate research that will ultimately elucidate the physiological mechanisms involved.

Conclusions

Our data shows that daytime sleepiness, but not circadian rhythms, correlated with microstructure of a brainstem sleep pathway connecting the suprachiasmatic nucleus and lateral medulla, providing the first evidence for a functional role of hypothalamic projections to the sympathetic nervous system in humans in sleep regulation.

Disclosure/Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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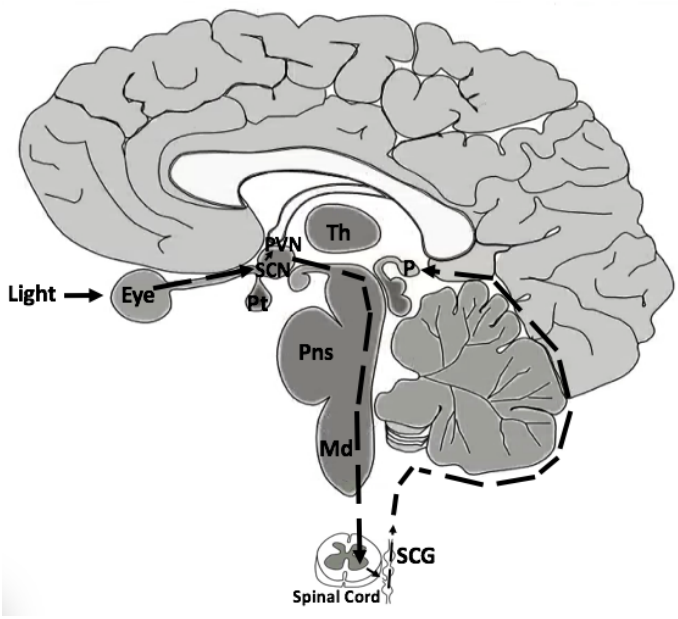


Figure 1. Multi-synaptic pathway from the suprachiasmatic nucleus to the pineal gland regulating light induced melatonin inhibition. Light signals are transduced by melanopsin-containing retinal ganglion cells in the eye and transmitted to the following structures in order: the suprachiasmatic nucleus (SCN), the paraventricular nucleus (PVN), the intermediolateral column of the thoracic spinal cord (via the lateral medulla) and the superior cervical ganglion, terminating in the pineal gland.

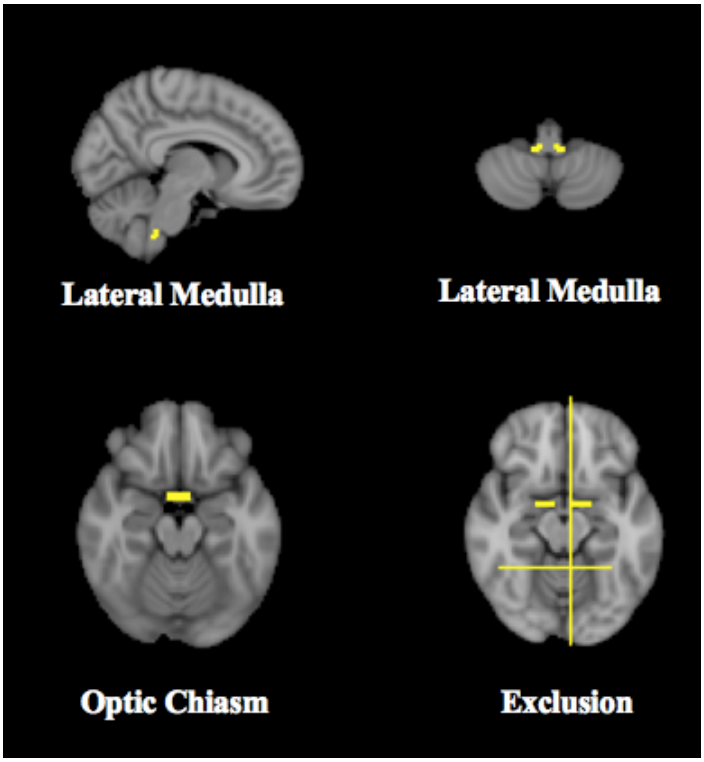


Figure 2. Region of interest masks used to virtually dissect connection between the suprachiasmatic nucleus and lateral medulla. Examples of lateral medulla masks are shown in the coronal (top left) and saggital (top right) planes. Optic chiasm masks are demonstrated in the axial plan (bottom left). An exclusion mask was used during dissection of all tracts and is shown in the axial plane (bottom right). The exclusion mask included regions that would ensure exclusion of the optic radiations, cerebellum and a cross over of connections towards the opposite hemisphere. Due to the close proximity of central descending pathways in the brain stem, the mid-saggital exclusion midline region was drawn either slight left or slight right, depending on the hemisphere in which the tract was dissected, in order to avoid exclusion of the entire tract. For example, the mid-saggital exclusion region represented in the bottom left above was used as exclusion to dissect tracts in the left hemisphere.

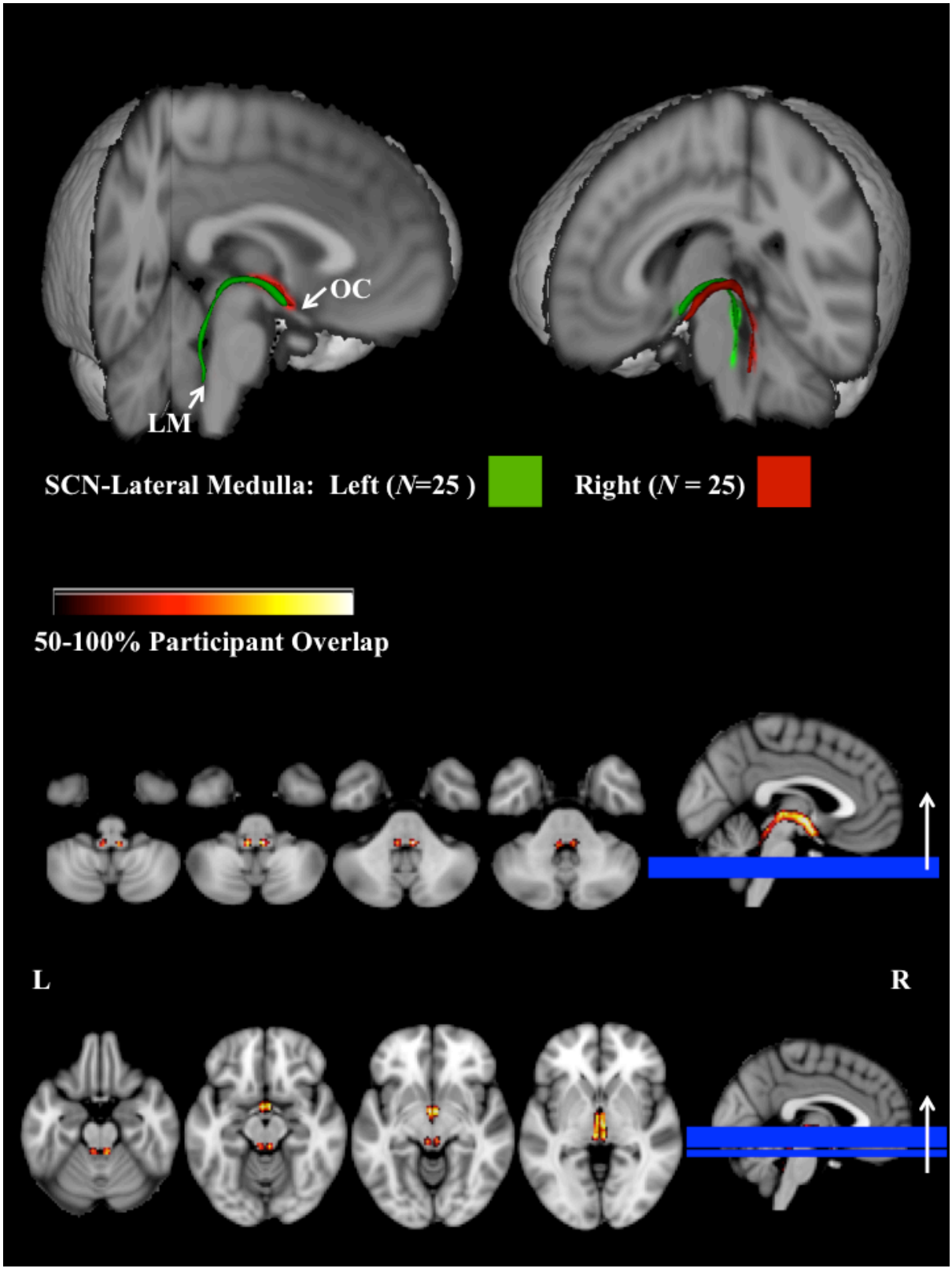
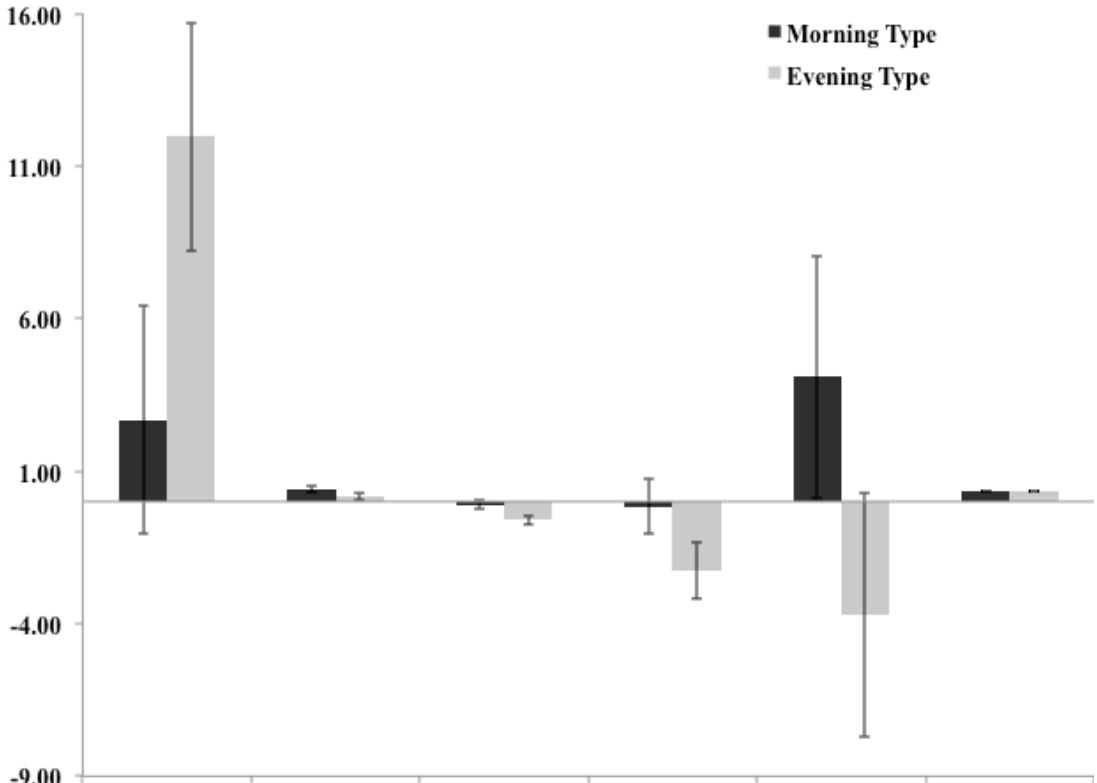


Figure 3. Probabilistic tractography between the suprachiasmatic nucleus and the lateral medulla (N = 25). Top: Composite 3D reconstruction of tracts in the left (shown in green) and right (shown in red) hemispheres across all participants, aligned to the Montreal

Neurological Institute T1-weighted standard brain. The composite connection was generated by adding connections of all participants together, and was thresholded to include only voxels that were common to connections in at least 75% of participants. Bottom: Composite tracts connecting suprachiasmatic nucleus-lateral medulla shown in consecutive inferior to superior axial slices. A lower threshold was used to include voxels that were common to connections in at least 50% of participants to demonstrate variability across participants.

Table 1. Fractional anisotropy (FA) values for suprachiasmatic nucleus-lateral medulla connections in the left and right hemispheres across morning and evening types of individuals. Participant 9 was excluded due to failure to experiment instructions.

Participant	MEQ Type	Left Tract	Right Tract
1	Definite Morning	0.36	0.37
2	Definite Morning	0.32	0.34
3	Moderate Morning	0.30	0.35
4	Definite Morning	0.31	0.32
5	Moderate Evening	0.27	-
6	Definite Evening	0.28	0.39
7	Definite Evening	0.33	0.24
8	Definite Morning	0.37	0.39
10	Moderate Morning	0.33	0.32
11	Definite Evening	0.35	0.38
12	Definite Morning	0.28	0.33
13	Definite Evening	0.29	0.32
14	Definite Morning	0.36	0.33
15	Moderate Morning	0.28	0.32
16	Moderate Morning	0.34	0.50
17	Definite Evening	0.28	0.35
18	Definite Evening	0.36	0.39
19	Definite Evening	0.32	0.31
20	Definite Evening	0.35	0.34
21	Moderate Morning	-	0.38
22	Definite Morning	0.28	0.35
23	Moderate Evening	0.34	0.30
24	Moderate Morning	0.34	0.36
25	Definite Morning	0.33	0.37
26	Moderate Morning	0.29	0.32
27	Moderate Morning	0.34	0.28
Average		0.32	0.34



	Melatonin pg/ ml	Cortisol µg/ml	Temperature °C	Breathing Rate (breaths/ min)	Heart Rate (beats/min)	FA
Morning Type	2.67	0.41	-0.10	-0.15	4.08	0.33
Evening Type	11.96	0.16	-0.60	-2.27	-3.73	0.32

Figure 4. Bar graphs demonstrating diurnal differences in measures of melatonin, cortisol, temperature, breathing rate and mean fractional anisotropy (FA) of the suprachiasmatic nucleus-lateral medulla connection between morning and evening type groups of individuals. Difference scores for melatonin, cortisol, temperature, breathing rate and heart rate were calculated by subtracting scores collected in the evening from scores collected in the morning. Therefore, for example, a positive value of melatonin in the evening type group indicates higher melatonin scores in the morning compared to the evening for evening types. A negative score indicates lower scores in the morning compared to the evening measure. Error bars represent standard error of the mean. Independent samples t-tests demonstrated a significantly larger difference only for temperature (($t(23) = 2.58$, $p = .017$), however this effect did not survive Bonferroni correction for multiple comparisons.

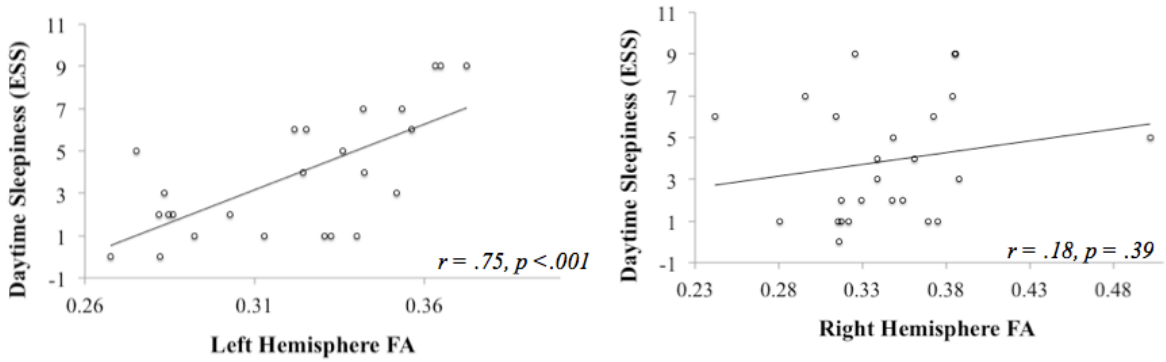


Figure 5. Correlation scatterplot demonstrating microstructure (FA) of suprachiasmatic nucleus-lateral medulla connection in the left hemisphere (left) predicted daytime sleepiness as measured by the Epworth Sleepiness Scale (Johns, 1991).

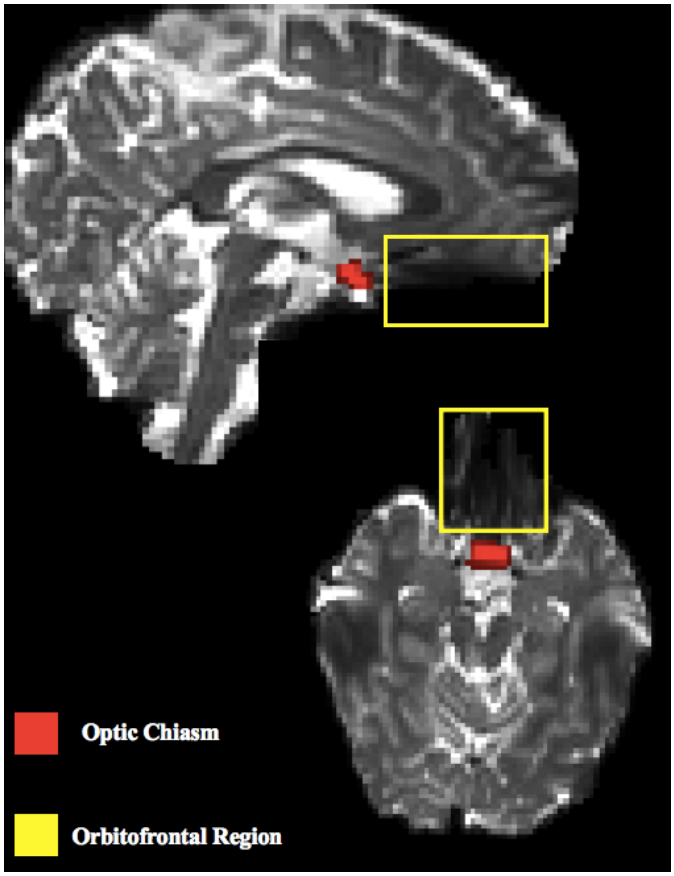


Figure 6. The figure above illustrates a non-diffusion (B0) image from one representative participant, with the optic chiasm and orbito-frontal cortex regions marked for comparison of distortions. As is seen on the image, the region affected marked with a yellow box shows dark distortion, not affecting the optic chiasm (marked with red mask).

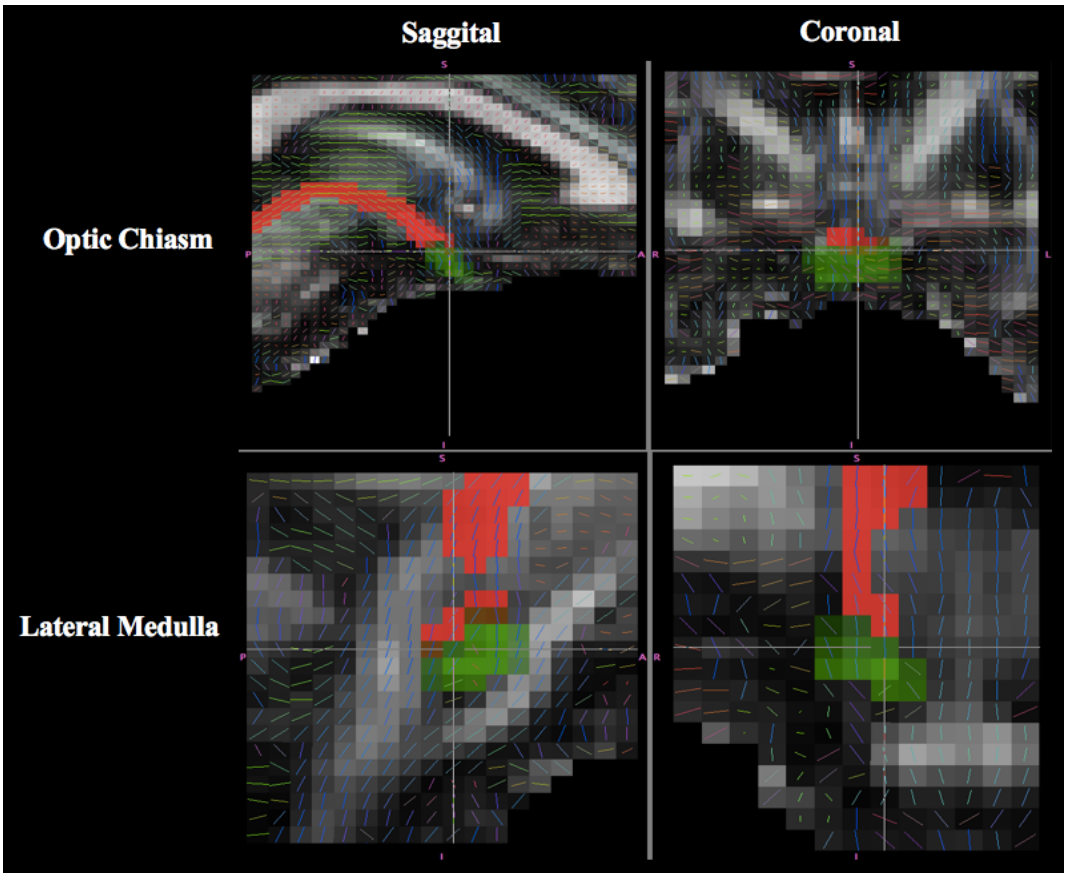


Figure 7. Close up illustration of tract (red) near the seed regions (green) in one representative subject (top: optic chiasm mask in green; bottom: lateral medulla mask in green; sleep tract in red. Note that the crosshairs highlight a voxel where the tract and mask overlap). As demonstrated, it can be seen that the direction in each voxel along the tract follow along a consistent trajectory. This can be clearly seen especially in the brainstem. The FA values are lower (~.2-.4), as expected, in regions where the streamline passes through gray matter in the mid-brain and medulla and higher as the tract passes through the brainstem and pons (~.5).